90. (**new**) The method of claim 48, 49 or 83, wherein the hairpin RNA is a chemically synthesized product.

91. (**new**) The method of claim 48, 49 or 83, wherein the hairpin RNA is a in vitro transcription product.

Remarks

In the course of the interview, the teachings of Agrawal PCT publication WO9401550 and Fire et al. US Patent 6,506,559 were discussed and distinguished from the methods of the pending claims. As requested by the Examiner, that discussion is summarized below.

At the time of the priority date of the present application, RNA interference (RNAi) had been demonstrated to be extremely active in several invertebrate species. However, while it may have been highly tempting to attempt to adapt this technology to mammalian cells, it had been demonstrated at that time that mammals, unlike the model systems of the prior art, had developed various protective phenomena against viral infections that impeded the use of RNA interference in mammalian cells.

For instance, it was know in the art that the presence of even extremely low levels of viral double-stranded RNA (dsRNA) triggers an interferon response (called "acute-phase response") and the activation of a dsRNA Responsive Protein Kinase (PKR). PKR phosphorylates and inactivates translation factor EIF2a leading to activation of the 2',5'-oligoadenylate synthetase, finally resulting in RNAse L activation. This cascade induces a global non-specific suppression of translation, which in turn triggers apoptosis. For review, see Williams (1997) "Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation" Biochem Soc Trans, 25(2):509-13. Consistent with this mechanism, the use of long dsRNA to silence expression in mammalian cells had been tried and reported at various meetings as being largely unsuccessful as a consequence to the constructs producing a general sequence-independent killing of the mammalian cells.

In several lower eukaryotes, an RNA-dependent polymerase is thought to amplify the introduced long dsRNA, possibly leading to higher levels of siRNAs. See, for example, Smardon et al.

(2000) "EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans" Curr. Biol. 10:169–178. In C. elegans, data support the idea that siRNA pairing with mRNA results in the extension of the siRNA along the mRNA template to produce a long dsRNA, which could then be processed by Dicer to produce even more siRNAs. However, at the time the present application was filed, it was believed that mammalian cells probably lack the siRNA amplification mechanisms that confer RNAi potency and longevity in organisms such as worms or plants. The criticality of this amplification mechanisms in the prior art model systems was not discerned, adding to the uncertainty as to whether RNAi would be possible in cultured cells of any type, let alone mammalian cells in culture or whole animals.

As described in the present application, the Applications have discovered that RNAi begins when a nucleolytic enzyme, referred to as DICER, encounters dsRNA and cleaves it into pieces called small-interfering RNAs or siRNAs. This protein belongs to the RNase III nuclease family. When incorporated into a larger, multicomponent complex named RISC (RNA-induced silencing complex) that includes Argonaut, the processed siRNAs form a "guide sequence" that targets the RISC to the desired mRNA sequence and promotes its destruction.

The use of hairpin RNA species to affect gene silencing in mammalian cells, according to the methods of the pending claims, is based on several factors that were first recognized by the Applicants. Among those factors are:

- mammalian cells contain the nucleolytic activity necessary to process hairpin RNA constructs and produce functional siRNA products;

- the use of short hairpin RNA constructs does not produce a general sequence-independent killing of the mammalian cells, e.g., does not trigger an acute-phase response and the activation of a PKR.

Moreover, as the examples of the present application demonstrate, there are certain unexpected benefits to using hairpin RNAs to cause RNA interference in mammalian cells. For instance, the hairpin RNA constructs generally exhibit better reassociation kinetics in cells than equivalent duplex RNA. Perhaps even more significant, the Applicants have demonstrated that transgenic cell lines can

be engineered to synthesize hair RNAs that exhibit a long-lasting suppression of gene expression that persists throughout cell divisions.

Agrawal PCT publication WO9401550

The Agrawal PCT publication is directed to antisense constructs, and as described below, does not teach or suggest nucleic acid constructs useful for producing RNA interference. To that end, Agrawal states, in the Summary of the Invention, "oligonucleotides according to the invention form stable hybrids with target sequences under physiological conditions, *activate RNase H* and produce only nucleosides as degradation products.....This results in oligonucleotides that activate RNase H, *an important feature for the antisense therapeutic compound.*" [emphasis added] Likewise, the Detailed Description section of the Agrawal application sets forth the requirements of the antisense construct that reference teaches, stating that "the invention provides therapeutic self-stabilized oligonucleotides that are *more resistant to nucleolytic degradation* than oligonucleotides that are known in the art", and "the invention provides nuclease resistant oligonucleotides that *activate RNase H*". [emphasis added]

RNase H degrades the RNA of DNA-RNA hybrids – which as Agrawal recognizes, is an important requirement for antisense constructs. However, RNase H does *not* degrade single-stranded nucleic acids, duplex DNA or double-stranded RNA. Thus, to the extent Agrawal teaches stabilized duplexes including RNA, those complexes must also include some further feature rendering them capable of activating RNase H. That is, in contrast to the hairpin RNA constructs of the pending claims, the stabilized hairpin constructs of the Agrawal reference cannot be ribonucleic acids alone.

In contrast, the hairpin RNAs of the pending claims do not work by a mechanism that would include activation of RNase H, and are specifically required to be susceptible to nucleolytic cleavage, e.g., as they must be substrate for cleavage by a RNaseIII enzyme. Accordingly, the teachings of Agrawal cannot be fairly interpreted to include the methods of the pending claims.

Those differences notwithstanding, Applicants note that the combination of short hairpin RNAs (e.g., such as having self complementary sequences of 19 to 100 nucleotides) for use in mammalian cells has several unexpected advantages not taught or suggested by the teachings of Agrawal. As described in further detail above and in the pending application, such hairpin constructs: can be

processed by mammalian cells to produce functional siRNA products; do not produce a general sequence-independent killing of the mammalian cells; exhibit favorable reassociation kinetics in cells; and can be used to engineer transgenic cell lines that exhibit a long-lasting suppression of gene expression that persists throughout cell divisions.

Fire et al. US Patent 6,506,559

The Fire et al. patent is directed to RNA interference, and teaches a variety of different constructs that are alleged to work in various cell-types, including mammalian cells. However, as set forth above, as of the priority date of the present application was filed, it was generally known in the art that the embodiments suggested from work in invertebrates, such as is the case of the Fire et al. patent, did not work in mammalian cells. Rather than induce sequence-dependent suppression, the long dsRNA constructs most favored in invertebrates induced sequence-independent cell death. In considering whether a reference qualifies as prior, it is respectfully noted that when a prior art reference merely discloses the structure of a compound, evidence showing that attempts to prepare that compound were unsuccessful before the date of invention will be adequate to show inoperability. In re Wiggins, 488 F.2d 538, 179 USPQ 421 (CCPA 1971).

The above allegations notwithstanding, Applicants note that the Fire et al. patent, like Agrawal, fails to teach or suggest the unexpected advantages arising from the combination of short hairpin RNAs for use in mammalian cells. Applicants contend that the combination of short hairpin RNAs for use in mammalian cells has several unexpected advantages not taught or suggested by the teachings of Fire et al. To reiterate, such hairpin constructs: can be processed by mammalian cells to produce functional siRNA products; do not produce a general sequence-independent killing of the mammalian cells; exhibit favorable reassociation kinetics in cells; and can be used to engineer transgenic cell lines that exhibit a long-lasting suppression of gene expression that persists throughout cell divisions.

Applicants believe no fees are due, however, if there are any fees due in connection with the filing of this Second Preliminary Amendment, please charge the fees to our **Deposit Account No. 18-1945.** Please direct any questions arising from this submission to the undersigned at (617) 951-7000.

Respectfully submitted,

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Marked-up version of claims showing changes made:

48. **(amended)** A method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (RNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions.

49. **(amended)** A method for attenuating expression of a target gene in mammalian cells, comprising introducing into the mammalian cells a single-stranded hairpin ribonucleic acid (RNA) comprising self complementary sequences of 19 to 100 nucleotides that form a duplex region, which self complementary sequences hybridize under intracellular conditions to a target gene, wherein said hairpin RNA (i) is cleaved in the mammalian cells to produce an RNA guide sequence that enters an Argonaut-containing complex, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) reduces expression of said target gene in a manner dependent on the sequence of said complementary regions.

50. **(amended)** The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the hairpin RNA is transfected into said mammalian cells.

51. **(amended)** The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the hairpin RNA is microinjected into said mammalian cells.

52. **(amended)** The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the hairpin RNA is a transcriptional product that is transcribed from an expression construct introduced into said mammalian cells, which expression construct comprises a coding sequence for transcribing said hairpin RNA, operably linked to one or more transcriptional regulatory sequences.

53. (reiterated) The method of claim 52, wherein said transcriptional regulatory sequences include a promoter for an RNA polymerase.

54. (reiterated) The method of claim 53, wherein said transcriptional regulatory sequences include a promoter for a bacteriophage RNA polymerase.

55. (reiterated) The method of claim 53, wherein said transcriptional regulatory sequences include a promoter for an cellular RNA polymerase.

56. (**amended**) The method of claim 53, wherein said promoter is selected from the group consisting of ~~a U6 promoter,~~ a T7 promoter, a T3 promoter, and an SP6 promoter.

57. (reiterated) The method of claim 52, wherein said transcriptional regulatory sequences includes an inducible promoter.

58. (reiterated) The method of claim 52, wherein said mammalian cells are stably transfected with said expression construct.

59. (reiterated) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein said hairpin RNA is a transcriptional product of an RNA-dependent RNA polymerase.

60. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the mammalian cells are germ line cells.

61. (**amended**) The method of claim 48, 49 or 83 ~~60~~, wherein the mammalian cells are stem cells.

62. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the mammalian cells are somatic cells.

63. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the mammalian cells are immortalized cells.

64. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the mammalian cells are primate cells.

65. (**amended**) The method of claim 64, wherein the primate cells are human cells.

66. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the mammalian cells are selected from the group consisting of adipocytes, fibroblasts, myocytes, cardiomyocytes,

endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

67. (reiterated) The method of claim 48 or 49, wherein the hairpin RNA is introduced into the mammalian cells in cell culture.

68. (reiterated) The method of claim 48 or 49, wherein the hairpin RNA is introduced into the mammalian cells in an animal.

69. (reiterated) The method of claim 48 or 49, wherein expression of the target is attenuated by at least 33 percent relative expression in cells not treated said hairpin RNA.

70. (reiterated) The method of claim 48 or 49, wherein expression of the target is attenuated by at least 90 percent relative expression in cells not treated said hairpin RNA.

71. (reiterated) The method of claim 48 or 49, wherein the target gene is an endogenous gene of the mammalian cell.

72. (reiterated) The method of claim 48 or 49, wherein the target gene is a heterologous gene relative to the genome of the mammalian cell.

73. (reiterated) The method of claim 48 or 49, wherein the target gene is a gene of a pathogen.

74. (reiterated) The method of claim 48 or 49, wherein the self complementary sequences hybridize under intracellular conditions to a mature mRNA transcript.

75. (reiterated) The method of claim 48 or 49, wherein the self complementary sequences hybridize under intracellular conditions to a non-coding sequence of the target gene.

76. (reiterated) The method of claim 48 or 49, wherein the self complementary sequences hybridize under intracellular conditions to an untranscribed sequence of the target gene, which untranscribed sequence is operably linked to the coding sequence of the target gene.

77. (reiterated) The method of claim 48 or 49, wherein the self complementary sequences hybridize under intracellular conditions to a non-coding sequence of the target gene selected from the group consisting of promoter sequence, enhancer sequence and intronic sequence.

78. (reiterated) The method of claim 48 or 49, wherein the self complementary sequences hybridize under intracellular conditions to a target gene selected from the group consisting of developmental genes, oncogenes, tumor suppressor genes, and genes encoding enzymes.

79. (reiterated) The method of claim 48 or 49, wherein the hairpin RNA includes one or more modifications to phosphate-sugar backbone or nucleosides residues.

80. (reiterated) The method of claim 79, wherein the modifications inhibit inactivation of the hairpin RNA by adenosine deaminase.

81. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the self complementary sequences are 20-50 nucleotides in length.

82. (**amended**) The method of claim 48, 49 or 83 ~~48 or 49~~, wherein the self complementary sequences are 29 nucleotides in length.

83. (**amended**) A method for attenuating expression of one or more target genes in mammalian cells, comprising introducing into the mammalian cells a variegated library of single-stranded hairpin ribonucleic acid (RNA) species, each hairpin RNA species comprising self complementary sequences of 19 to 100 nucleotides that form duplex regions and which hybridize under intracellular conditions to a target gene, wherein each of said hairpin RNA species (i) is a substrate for cleavage by a RNaseIII enzyme to produce a double-stranded RNA product, (ii) does not produce a general sequence-independent killing of the mammalian cells, and (iii) if complementary to a target sequence, reduces expression of saida target gene in a manner dependent on the sequence of said complementary regions.

84. (reiterated) The method of claim 83, wherein said variegated library of hairpin RNA species collectively attenuate expression of a plurality of different target genes.

85. (reiterated) The method of claim 83, wherein said variegated library of hairpin RNA species are arrayed a solid substrate.

86. (reiterated) The method of claim 83, wherein said variegated library of hairpin RNA species are arrayed in wells of a multi-well plate.

87. **(new)** The method of claim 83, including the further step of identifying hairpin RNA species of said variegated library which produce a detected phenotype in said mammalian cells.

88. **(new)** The method of claim 55, wherein said promoter is an RNA polymerase III promoter or an snRNA promoter.

89. **(new)** The method of claim 88, wherein said promoter is an U6 promoter.

90. **(new)** The method of claim 48, 49 or 83, wherein the hairpin RNA is a chemically synthesized product.

91. **(new)** The method of claim 48, 49 or 83, wherein the hairpin RNA is a in vitro transcription product.